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Nerve Growth Factor-Induced Derepression of Peripherin Gene Expression Is Associated with Alterations in Proteins Binding to a Negative Regulatory Element

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The peripherin gene, which encodes a neuronal-specific intermediate filament protein, is transcriptionally induced with a late time course when nerve growth factor (NGF) stimulates PC12 cells to differentiate into neurons. We have studied its transcriptional regulation in order to better understand the neuronal-specific end steps of the signal transduction pathway of NGF. By 5′ deletion mapping of the peripherin promoter, we have localized two positive regulatory elements necessary for full induction by NGF: a distal positive element and a proximal constitutive element within 111 bp of the transcriptional start site. In addition, there is a negative regulatory element (NRE; −179 to −111), the deletion of which results in elevated basal expression of the gene. Methylation interference footprinting of the NRE defined a unique sequence, GGCAGGGCGCC, as the binding site for proteins present in nuclear extracts from both undifferentiated and differentiated PC12 cells. However, DNA mobility shift assays using an oligonucleotide probe containing the footprinted sequence demonstrate a prominent retarded complex in extracts from undifferentiated PC12 cells which migrates with slower mobility than do the complexes produced by using differentiated PC12 cell extract. Transfection experiments using peripherin-chloramphenicol acetyltransferase constructs in which the footprinted sequence has been mutated confirm that the NRE has a functional, though not exclusive, role in repressing peripherin expression in undifferentiated and nonneuronal cells. We propose a two-step model of activation of peripherin by NGF in which dissociation of a repressor from the protein complex at the NRE, coupled with a positive signal from the distal positive element, results in derepression of the gene.

Nerve growth factor (NGF) is a peptide that is required for the differentiation and maintenance of sympathetic and sensory neurons of the peripheral nervous system (30, 43, 44) as well as cholinergic neurons of the central nervous system (54, 69). NGF is one of the environmental factors which influence cells derived from the sympathoadrenal sublineage of the neural crest to differentiate into sympathetic adrenergic neurons when they reach their peripheral point of migration (3, 15, 39, 64). This developmental decision can be studied in vitro with PC12 cells, a cell line derived from a rat pheochromocytoma which behaves similarly to pluripotent neural crest cells (27, 28, 66). After approximately 18 h of exposure in culture to NGF, PC12 cells begin to extend neurites and acquire biochemical and membrane properties of sympathetic neurons (reviewed in reference 28).

Recently, knowledge of early events in the NGF signal transduction pathway has increased dramatically with the discovery that the *trk* proto-oncogene, a receptor with tyrosine kinase activity, may participate in formation of the high-affinity NGF receptor (29, 33, 36). NGF quickly stimulates several other cytoplasmic protein kinases (37, 71). Treatment of PC12 cells with NGF rapidly and transiently activates the transcription of a set of genes termed immediate-early genes (reviewed in reference 65), including c-*fos* (12, 24, 51), c-*jun* (6), NGF1-A (52), and NGF1-B (53). The transcriptional activation of these genes is protein synthesis independent (25), in contrast to the activation of the delayed-early genes, which occurs within 1 to 2 h of NGF treatment (21). Tyrosine hydroxylase, one such delayed-early gene, is regulated by c-*fos* and c-*jun* (21). The third level in the cascade of gene activation, so-called late genes (reviewed in reference 65), consists of genes whose induction occurs hours to days after initiation of NGF treatment, coincident with the acquisition of the neuronal phenotype. Leonard et al. (41, 42) identified four such genes, the peripherin, thymosin β4, clone 63 (identified as calcyclin [unpublished data]), and clone 71 genes, in a differential screening of cDNA libraries representing mRNA from untreated PC12 cells and PC12 cells treated for 2 weeks with NGF. Other laboratories have reported similar kinetics of induction by NGF for NF-L and NF-M (45), SCG10 (66), and the brain type II sodium channel (47), among others. Conceivably, the induction of late genes could be dependent on transcription factors encoded by genes induced in the first or second wave of gene activation.

The steps in the NGF signal transduction pathway which trigger neuronal differentiation are poorly understood. Induction of immediate-early genes is a general response to growth factors in diverse cell types (e.g., serum stimulation of fibroblast cells [10, 26, 40]). Likewise, NGF and epidermal growth factor (EGF) activate the same set of 10 immediate-early genes (6), although EGF does not induce neuronal differentiation (7). There is great interest in deter-

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mining the steps after this general response which lead to neuronal-specific gene activation.

We have chosen to study the regulation of peripherin gene expression during NGF-induced neuronal differentiation as an approach to answering how the neuronal-specific signal is transmitted by the NGF signal transduction pathway. The peripherin gene is a late gene which is expressed approximately 12 h after initiation of NGF treatment of PC12 cells (41, 42), corresponding to the time at which PC12 cells begin to express a neuronal phenotype (28). Peripherin mRNA levels are selectively induced by NGF and fibroblast growth factor and are only minimally increased after EGF exposure (<2-fold [42]). The peripherin gene encodes a neuronal-specific type III intermediate filament protein, first described by Portier et al. (57), which is expressed in sympathetic, parasympathetic, and sensory ganglia of the peripheral nervous system and by a small subset of neurons in the central nervous system (42, 56). During development, it is first expressed by day 11.5 of embryogenesis in the rat (days 9 to 9.5 of embryogenesis in the mouse) (16, 22, 68), corresponding to the time at which sympathetic ganglia first begin to form (1). Therefore, expression of the peripherin gene is closely associated with the terminally differentiated neuronal phenotype.

Previously, we have reported the cloning of the rat gene encoding peripherin and the sequencing of the peripherin promoter (67). In this report, we describe the mapping of regulatory regions of the peripherin promoter which contribute to the NGF-induced and neuronal-specific expression of peripherin. In particular, we have focused on a negative regulatory element (NRE) which contributes to the repression of peripherin expression in undifferentiated PC12 cells and nonneuronal cell types. We have shown by DNA mobility shift assays that the proteins complexed to the NRE are altered as a result of NGF treatment, suggesting that NGF-mediated events cause the release of a repressor protein from this site.

MATERIALS AND METHODS

Cell culture and treatment. Stock cultures of PC12 cells were maintained as described previously (21). PC12 cells grown for nuclear extract preparation were plated on plastic culture dishes coated with collagen (Collagen Corp., Palo Alto, Calif.) at a density of $\approx 3 \times 10^6$ cells per 150-mm dish. NGF was added 24 h after plating (50 ng/ml; rat β-NGF purchased from Robert Stack, University of Michigan, Flint). NIH 3T3 cells were maintained in Dulbecco modified Eagle medium containing 10% defined and supplemented calf serum (HyClone, Logan, Utah). The clone 9 liver cell line was generously provided by William Dolan (New York University Medical Center) and was maintained in F-12 medium (GIBCO-BRL, Bethesda, Md.) and 10% calf serum (HyClone).

Nuclear run-on transcription assay. Experimental procedures for isolation of nuclei and the in vitro nuclear run-on transcription assay have been described previously (24). The method for binding 5 μg of linearized plasmid to nitrocellulose was performed as described by Greenberg and Ziff (26). Sources of cloned plasmid DNAs were as follows: pv-*fos* (*fos* [20]), pclone 73 (peripherin), and pclone 2 (42).

Plasmid constructions and mutagenesis. Initially, a 3.85-kb *Eco*RI-*Sma*I fragment of peripherin genomic DNA was subcloned into pUC-18 (p5PB6). The *Eco*RI site was cut, blunt ended with Klenow enzyme, and ligated to *Hin*dIII linkers. To construct the −3710-CAT plasmid, the *Hin*dIII-*Sma*I 3.85-kb fragment was ligated into the polylinker site just 5′ of the DNA segment coding for the bacterial enzyme chloramphenicol acetyltransferase (CAT) in a pUC-13-derived vector. The resulting hybrid peripherin-CAT plasmid includes peripherin sequences −3710 to +142 relative to the peripherin transcriptional start site. Since this includes 87 bp of peripherin coding sequence, the junction of peripherin and CAT DNA was sequenced to ensure continuity of the two reading frames. The −2660-CAT and −988-CAT plasmids were constructed by ligating *Sal*I-*Xma*I and *Acc*I-*Xma*I peripherin DNA fragments, respectively, into the polylinker site of the pUC-CAT vector. (The *Xma*I site coincides with the *Sma*I site and produces an identical peripherin-CAT junction). Plasmids −2290-CAT, −2080-CAT, and −179-CAT were constructed by subcloning fragments generated by Bal 31 digestion of the −2660-CAT plasmid linearized at the *Sal*I site, following the procedure of Maniatis et al. (48). Basically, the Bal 31-digested ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I, and the peripherin sequence was released from the plasmid with *Xma*I. The pUC-CAT vector was linearized in the polylinker site with *Sal*I, further digested with *Xma*I after the *Sal*I site was filled in, and ligated to the Bal 31 peripherin fragments. The Bal 31 peripherin fragments were also subcloned into M13 for dideoxynucleotide sequencing (61) to determine the 5′ endpoint of the Bal 31 digestion.

The polymerase chain reaction was used to produce the peripherin sequence in plasmids −520-CAT, −305-CAT, and −111-CAT. 5′ primer oligonucleotides contained a sequence identical to the appropriate peripherin promoter coding strand and additional nucleotides, creating a *Hin*dIII restriction site. The 3′ primer oligonucleotide sequence, common to all three reactions, was complementary to peripherin coding strand sequences and included the *Xma*I sequence at +142. The template used for amplification was the 1-1-Bλ phage containing peripherin genomic DNA (67), linearized with *Eco*RI and *Nco*I. Reactions were run with *Taq* polymerase (Perkin-Elmer Cetus) for 25 amplification cycles in a DNA thermal cycler (Perkin-Elmer Cetus) as described by Saiki et al. (59). Reaction products were cut with *Hin*dIII and *Xma*I, gel purified, and subcloned into pUC-CAT. For each construct, the subclone that was grown up was digested with *Hin*dIII and *Xma*I, and the insert was cloned into M13 and sequenced to exclude the possibility of errors introduced by *Taq* polymerase.

Plasmids with mutations in the putative repressor binding site were generated by oligonucleotide-mediated, site-directed mutagenesis according to the method of Kunkel (38). Briefly, a 447-bp *Hin*dIII-*Xma*I DNA fragment containing the peripherin sequences (−305 to +142) in the −305-CAT plasmid was subcloned into M13mp19 and grown in *E. coli* RZ1032 *dut ung*. In vitro mutagenesis was then performed with the synthetic oligonucleotide 5′-CTCCTGGTTGCTA ACGCCCTACTTGCTTGATTCCC-3′ (which corresponds to the RM3 sequence in Fig. 4A), and the replicative-form recombinants were grown in *E. coli* TG1 (48). The *Hin*dIII-*Xma*I DNA fragment (−305 to +142) was subcloned back into pUC-CAT to generate mut-305-CAT. A *Bam*HI-*Xma*I DNA fragment (−245 to +142) was subcloned into −3710-CAT digested with *Xma*I and *Bam*HI under partial digestion conditions to generate mut-3710-CAT. The mutation and surrounding sequence were confirmed by double-stranded dideoxy sequencing.

A plasmid for detecting exogenous peripherin-CAT mRNA transcripts in transfection experiments by RNase protection analysis was constructed as follows: a *Bam*HI-

*Dra*I fragment was excised from the −988-CAT peripherin-CAT hybrid construct and cloned into the pGEM-3 vector digested in the polylinker region at *Bam*HI and *Sma*I. This fragment contains a sequence corresponding to −245 to +142 of the peripherin promoter in addition to the first 167 bp of the CAT gene sequence. The resulting plasmid is referred to as (P-C)pGEM.

Nuclear extract preparation and gel retardation assays. PC12 nuclear extracts were prepared from unstimulated and NGF-stimulated cell cultures grown as described above. Nuclear extracts from PC12 cells and clone 9 liver cells were prepared essentially as described by Dignam et al. (14), using a buffer containing 0.42 M KCl to extract nuclear proteins. The nuclear extracts were dialyzed for 4 h against BC100 buffer (0.02 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM NaF) at 4°C, aliquoted, and frozen at −70°C. Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad, Richmond, Calif.). Nuclear extracts from mouse erythroleukemia (MEL) cells (untreated or treated with HMBA [hydroxymethyl bis acetimide]), which were prepared in a similar manner, were generously provided by Larry Kenyon (New York University Medical Center).

For DNA-protein binding reactions, 40,000 cpm (≈0.2 ng) of kinase-treated ^{32}P-labelled oligonucleotide was mixed on ice with 5 μg of poly(dI-dC) (Pharmacia-LKB Technology, Inc., Piscataway, N.J.), 100 ng of nonspecific single-stranded oligonucleotide, BC100 without glycerol (10 μl), 1 mM $MgCl_2$, and 20 μg of PC12 nuclear extract (total reaction volume of 30 μl). The binding reaction was allowed to take place for 30 min at room temperature, conditions similar to those of Carthew et al. (8). Competition experiments included 150 ng of unlabelled, double-stranded oligonucleotide or DNA fragments in the reaction mixture (greater than 100 molar excess). DNA-protein complexes were resolved on 5.0% polyacrylamide gels (30:0.8, acrylamide/bisacrylamide), using conditions described previously (20).

Transient cell transfections and CAT assays. Transfection of plasmids into PC12 cells was performed by electroporation (18). Briefly, PC12 cells were grown to ≈70% confluence, and fresh medium was given to cells 24 h prior to the day of transfection. Cells were harvested as cell pellets containing 3×10^7 cells for each DNA sample used in transfection. Equimolar amounts of supercoiled peripherin-CAT plasmids were used, with 25 μg used for the shortest deletion construct, −111-CAT. In certain experiments, 5 μg of Rous sarcoma virus (RSV)–β-galactosidase plasmid was cotransfected. Electroporation was performed with an Isco model 494 power supply as previously described in Gizang-Ginsberg and Ziff (21). After electroporation, cells in each electroporation cuvette were distributed to four collagen-coated 10-cm dishes containing medium. Routinely, two plates were stimulated with 50 ng of NGF per ml beginning 2 h after plating, for a total of 46 h of exposure, while two plates were unstimulated. Cells were harvested for RNA or protein 48 h after electroporation, and duplicated plates were pooled. 3T3 cells were plated at a density of 10^6/10-cm plate 24 h prior to transfection and were given fresh medium 3 h prior to transfection. Equimolar amounts of supercoiled plasmid DNAs were used (20 μg for −111-CAT), and transfection was performed by calcium phosphate precipitation (70). Fresh medium was added to the cells 16 h after transfection, and cells were harvested 48 h after transfection.

Protein extracts were obtained by repeated freeze-thawing (three times) of cells suspended in 0.25 M Tris, pH 7.8. A portion of the protein extract was heated at 65°C for 10 min to inactivate endogenous deacetylases. The protein concentration of aliquots of unheated and heated protein extract was determined by using the Bio-Rad protein assay kit. CAT enzymatic assays were performed as described by Gorman et al. (23), using equal amounts of heated protein extract and a 2-h incubation at 37°C. β-Galactosidase enzymatic assays were performed as described by An et al. (2), using equal amounts of unheated protein extract.

RNase protection analysis. For RNase protection assays, RNA was obtained from transfected cells by the hot acid-phenol method (58). RNA obtained from the final precipitation was treated with RNase-free DNase (5 μg/ml; Bethesda Research Laboratories, Gaithersburg, Md.). Hybrid peripherin-CAT mRNA transcripts, as well as endogenous peripherin mRNA transcripts, were detected by the RNase protection method essentially as described by Melton et al. (50). RNA (25 μg) was hybridized with a ^{32}P-labelled antisense RNA probe synthesized from the T7 promoter of the (P-C)pGEM plasmid linearized with *Bam*HI. RNase-resistant fragments were separated on 6% polyacrylamide–8 M urea gels and visualized by autoradiography. ^{32}P-labelled *Hae*III-digested pBR322 DNA fragments were used as approximate size markers.

Methylation interference footprinting. Methylation interference footprinting was performed with the *Bam*HI-*Sty*I 147-bp restriction fragment spanning −245 to −98 of the peripherin promoter. The probe was asymmetrically radiolabelled with polynucleotide kinase at either the *Bam*HI site or the *Sty*I site to footprint the coding or noncoding DNA strand, respectively. The methylation interference footprinting was performed essentially as described by Baldwin (4) except that after the partially methylated DNA was used as probe in a DNA mobility shift reaction, DNA was eluted from the bands representing bound and free DNA by electroelution onto NA45 paper (Schleicher & Schuell, Keene, N.H.), using a Bio-Rad mini-Transblot apparatus. DNA eluted from each of the retarded bands obtained yielded a similar methylation interference pattern; thus, for the experiment depicted in Fig. 3, bound DNA was eluted from the prominent slowly migrating band in the undifferentiated nuclear extract and from the stronger of the two doublet bands in the differentiated nuclear extract. Cleavage fragments resulting from piperidine treatment of the methylated DNA were loaded onto an 8% polyacrylamide–urea sequencing gel. Maxam and Gilbert G-specific and purine-specific reactions were run for orientation (48).

Synthetic DNA oligonucleotides. Oligonucleotides used in DNA mobility shift assays were synthesized on an Applied Biosystems automatic DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis (PAGE). Certain oligonucleotides (WT [wild type], RM1, and RM2) were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, Tex.) and were also purified by PAGE.

RESULTS

NGF induces peripherin expression at the level of transcription. To determine whether the peripherin gene is induced by NGF at the level of transcription, we used a nuclear run-on transcription assay (24) to measure the transcription rate of peripherin at various times after initiating NGF treatment of PC12 cells. The results (Fig. 1) indicate that the peripherin gene is transcriptionally activated within 12 h of NGF

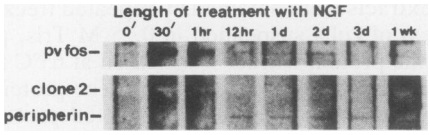

FIG. 1. Transcriptional activation of the peripherin gene in NGF-stimulated PC12 cells. A nuclear run-on transcription assay was performed as described previously (24). ^{32}P-labelled RNA transcripts were prepared by using nuclei isolated from PC12 cells treated for various times with NGF (50 ng/ml). The radiolabelled RNA was hybridized to the indicated linearized plasmid DNAs (5 μg), which were immobilized on nitrocellulose filters. Radioactivity was visualized by autoradiography. Clone 2 is a cDNA isolated from PC12 cells for which no transcriptional activation is observed and serves as a negative control. ′, minutes; d, days.

treatment, and transcription continues in cells which have differentiated in response to at least 1 week of NGF exposure. This pattern of transcriptional activation is in contrast to that of c-*fos* and of the tyrosine hydroxylase gene, which are transiently induced within 30 min (24) and 1 h (21), respectively, after NGF treatment.

5′ deletion mapping of the peripherin promoter. To identify regulatory elements in the peripherin promoter which control the NGF-induced expression of peripherin, we performed deletion mapping of the 5′ flanking region of the peripherin genomic sequence. We first constructed a hybrid plasmid in which a 3.85-kb genomic restriction fragment containing peripherin sequences from −3710 to +142 was fused to a promoterless bacterial CAT structural gene in a plasmid containing the simian virus 40 3′ polyadenylation signal sequence. When the peripherin-CAT plasmid was introduced by transient transfection into untreated PC12 cells or into PC12 cells subsequently treated for 46 h with NGF, CAT expression was 8.25-fold (σ = 1.4) higher in the NGF-treated cells (Fig. 2A). This degree of induction correlates well with the level of induction of the endogenous gene by NGF (42). As with the endogenous peripherin gene (42), there is a small basal level of activity of the peripherin-CAT construct in untreated PC12 cells. The 46-h length of NGF treatment was chosen as the longest possible within the time constraints of transient transfection assays. The effect of NGF on reporter gene transcription is clearly apparent at this time point. In parallel transfections, CAT expression from control plasmids in which the CAT reporter gene was driven by the RSV promoter increased by a factor of 1.4 in the presence of NGF, while expression from a thymidine kinase-CAT plasmid showed no increase in the presence of NGF (data not shown). Therefore, 3.85 kb of peripherin 5′ flanking DNA contains regulatory sequences which specifically confer NGF inducibility on the heterologous CAT gene.

A series of test plasmids with progressive deletions in the peripherin 5′ flanking sequence was constructed by using either available restriction sites, Bal 31-generated random deletions, or polymerase chain reaction cloning (see Materials and Methods). Figure 2A demonstrates the results of transient transfections divided into parallel plates of PC12 cells with or without subsequent treatment with NGF. Deletion of the 5′ boundary of peripherin sequence from −3710 to −2660 results in a drop of 57% in the NGF-induced activity of the CAT reporter gene; however, this promoter construct is still induced sixfold by NGF. Further deletion to −2290 results in a decrease to 5% of the NGF-induced activity of the −3710-CAT construct and a concomitant reduction in NGF-inducibility to threefold. A small increase in both NGF-induced and basal CAT activity occurs with deletion to 520 and 305 remaining nucleotides of peripherin promoter sequence. Most importantly, deletion from −179 to −111 of the peripherin promoter results in a 26-fold increase in NGF-induced CAT activity and a 16-fold increase in basal CAT activity. CAT activity resulting from transfection of the minimal construct (−111-CAT) into PC12 cells in the presence of NGF is similar (95%) to that of the −3710-CAT construct, and significantly, the basal level of expression of −111-CAT is threefold higher than that of −3710-CAT.

RNase protection assays were performed to demonstrate that the changes in CAT activity resulting from progressive deletion of the peripherin promoter sequences reflect changes in mRNA levels rather than alterations in translation of CAT RNA. A ^{32}P-labelled hybrid peripherin-CAT RNA probe spanning the peripherin start site (Fig. 2B) was used to detect RNA both from the transfected plasmid (309 bp) and from the endogenous peripherin gene (142 bp). The results (Fig. 2B) reveal the same pattern of expression from the peripherin-CAT constructs as seen by CAT assay. Also, the size of the protected fragments confirm that transcription begins at the bona fide peripherin transcriptional start site. There is evidence for a low level of spurious transcription from the minimal (−111) construct (lanes 10 and 11).

5′ deletion mapping defines a negative regulatory element as well as distal and proximal positive elements. These data suggest that a positive element necessary for induction of peripherin transcription by NGF is located between −2660 and −2290 and that full activity is enhanced by additional sequences between −3710 and −2660 of the peripherin 5′ flanking DNA. The sudden increase in basal as well as NGF-induced activity upon deletion from −179 to −111 strongly suggests that (i) an NRE is present between −179 and −111 and (ii) a constitutively active positive promoter element(s) is present 3′ of −111. Preliminary data place a necessary positive element between −80 and −40 in the peripherin promoter (data not shown). Taken together, these results suggest the model that a repressor protein binding to an NRE (−179 to −111) functions to inhibit basal expression of peripherin unless repression is relieved by binding of an NGF-induced positive factor to an upstream element (−2660 to −2290).

Preferential expression of peripherin constructs in PC12 cells. To assess whether the 3,710 bp of 5′ flanking sequence contains the regulatory elements necessary for the neuronal-specific expression of peripherin, expression of the −3710-CAT construct in NIH 3T3 cells (a mouse fibroblast cell line) was compared with its expression in PC12 cells. In addition, the −111-CAT construct was transfected into 3T3 cells. To correct for differences in transfection efficiency between cell lines, the RSV–β-galactosidase plasmid was cotransfected as an internal control, and CAT activity was normalized to β-galactosidase activity in protein extracts from the transfected cells. This standardization assumes that the RSV promoter is equally active in PC12 cells (with and without NGF) and 3T3 cells. The level of expression of the −3710-CAT construct in 3T3 cells is 4-fold lower than its expression in undifferentiated PC12 cells and 25-fold lower than its expression in NGF-differentiated PC12 cells (Fig. 2C). Therefore, regulatory sequences in the 3,710 bp of the 5′ flanking sequence confer a preferential expression of peripherin in neuronally differentiated cells. However, absolute tissue specificity must depend on additional sequences elsewhere in the peripherin gene, since there is a small but

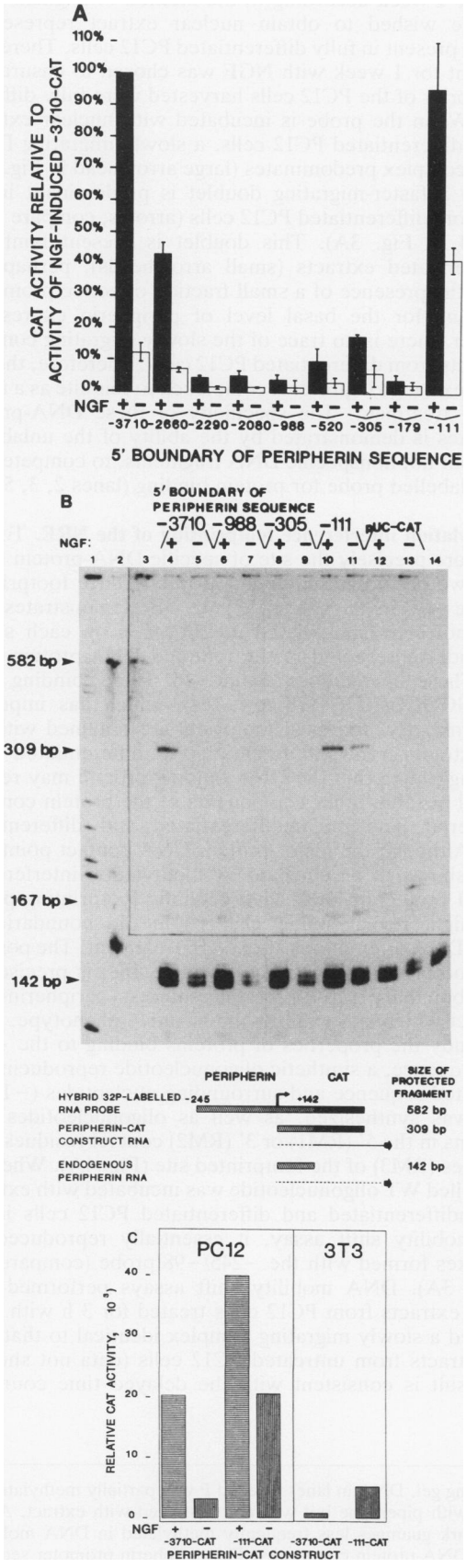

FIG. 2. Deletion mapping of the peripherin promoter. A series of hybrid peripherin-CAT plasmids was constructed as detailed in Materials and Methods. The 5′ boundary of the peripherin sequence is indicated, and the 3′ boundary is consistently +142, relative to the peripherin transcriptional start site. (A) Equimolar amounts of plasmid DNA were transfected by electroporation into PC12 cells, and parallel plates were either untreated or treated with NGF for a total of 46 h. For each experiment, the CAT activity of each deletion construct was expressed as a percentage of the CAT activity of the −3710-CAT construct in NGF-treated cells. For each construct, the results of at least two experiments were averaged, and the mean is shown in the histogram. The error bars indicate the range of the most disparate results. (B) RNase protection assay (see Materials and Methods). The schematic diagram shows the ^{32}P-labelled RNA probe which is transcribed from a *Bam*HI-*Dra*I restriction fragment obtained from a hybrid peripherin-CAT plasmid and cloned into pGEM-3. This sequence spans the peripherin transcriptional start site (indicated by an arrow) and the junction of peripherin (cross-hatched) and CAT (black) sequences. Therefore, RNA transcribed from the transfected hybrid peripherin-CAT plasmids (dashed and solid thin line) protects peripherin and CAT sequences of the RNA probe (309 bp), whereas RNA transcribed from the endogenous peripherin gene (dashed thin line) protects only the peripherin portion of the probe (142 bp). Lanes: 1, ^{32}P-labelled pBR322-*Hae*III size markers; 2, undigested ^{32}P-labelled RNA probe; 4 to 13, 25 μg of RNA prepared from PC12 cells transfected with either hybrid peripherin-CAT plasmids or with pUC-CAT, a promoterless CAT plasmid; 3 and 14, control lanes containing the ^{32}P-labelled probe hybridized with 20 μg tRNA (lane 3) and with 10 μg of RNA from untransfected PC12 cells treated with NGF for 1 week (lane 14). The area to the right of and above the black line represents an autoradiograph of the gel exposed for 3 days, whereas the area to the left and below the line is a 7-h exposure of the same gel to avoid overexposure of the bands representing probe protected by the endogenous peripherin gene. (C) Equimolar amounts of test plasmids were cotransfected with 5 μg of an RSV-β-galactosidase internal control plasmid into PC12 cells (via electroporation) or 3T3 cells (via calcium phosphate precipitation). In PC12 cell transient transfections, parallel plates were untreated or treated with NGF for a total of 46 h. Differences in transfection efficiency between the two cell types are corrected for by normalization to β-galactosidase activity in the protein extracts from the transfected cells. Units of normalized activity are calculated as (percent CAT activity per microgram of heated protein assayed per picomole of test DNA transfected)/(units of β-galactosidase activity per microgram of protein assayed per microgram of RSV-β-galactosidase plasmid transfected). Results represent the average of at least two experiments, with less than 25% variation between experiments.

significant level of expression in 3T3 cells. Deletion to −111 bp of the peripherin promoter sequence results in a sevenfold increase in activity in 3T3 cells, suggesting that peripherin expression in 3T3 cells is partially inhibited by negative regulatory sites upstream from −111. However, the level of expression of the −111-CAT construct is still fourfold less than the level of expression of the same construct in untreated PC12 cells, indicating that the proximal promoter is not as active in nonneuronal cells as in PC12 cells.

Proteins binding to the NRE are altered during neuronal differentiation of PC12 cells. If the NRE defined by 5′ deletion mapping is important in keeping peripherin expression low in the undifferentiated PC12 cell, then regulatory proteins complexed to this element would be expected to change upon NGF-induced differentiation. To test this hypothesis, we performed a DNA mobility shift assay using as probe a ^{32}P-labelled restriction fragment (−245 to −98) which spanned the boundaries of the deletions defining the NRE (Fig. 3A). Nuclear extracts were prepared from either untreated PC12 cells or PC12 cells grown in the presence of

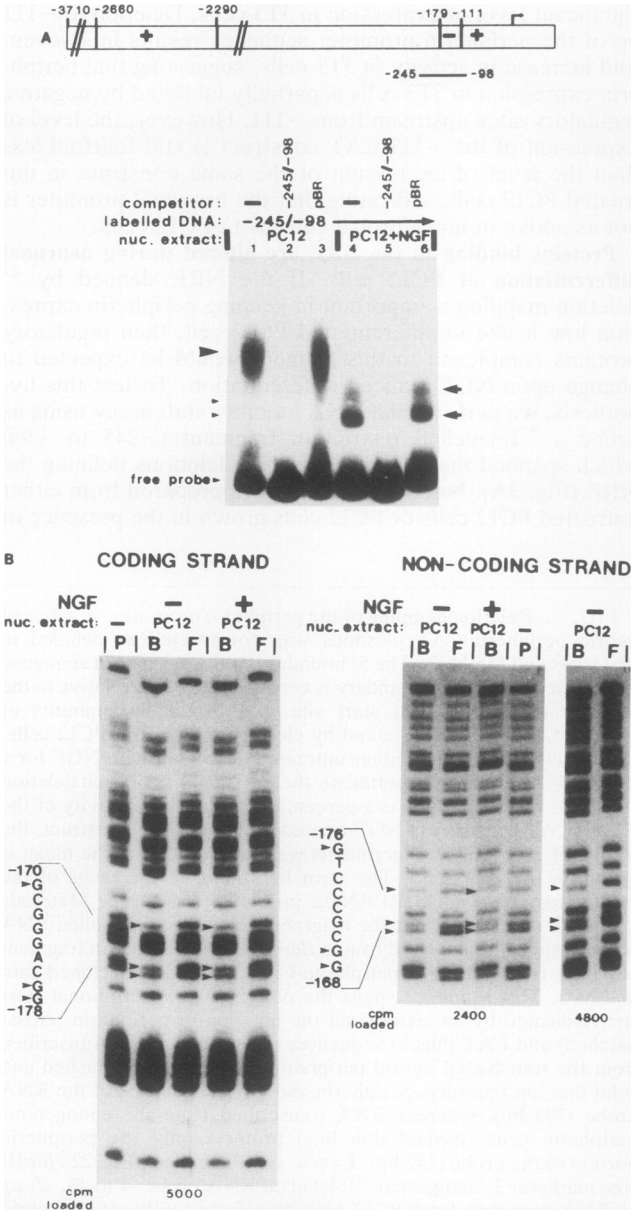

FIG. 3. Proteins bind specifically to the NRE and are altered by NGF treatment of PC12 cells. (A) The schematic of the peripherin promoter shows the positive and negative regulatory elements defined by 5′ deletion mapping. The NRE is defined by the deletions whose 5′ boundaries are −179 and −111. The dark line below the schematic represents the *Bam*HI-*Sty*I restriction fragment used as a probe in the DNA mobility shift assay and methylation interference footprinting. Nuclear extracts were prepared by the method of Dignam et al. (14) from untreated PC12 cells or PC12 cells grown in the presence of NGF for 1 week. DNA mobility shift assays were performed with the ^{32}P-labelled −245 to −98 fragment of the peripherin promoter, in the presence of no competitor DNA (lanes 1 and 4) or in the presence of −245/−98 peripherin DNA (lanes 2 and 5) or unlabelled pBR322 DNA digested with *Hae*III (lanes 3 and 6). (B) The −245/−98 *Bam*HI-*Sty*I peripherin fragment was asymmetrically end labelled and partially methylated with dimethyl sulfide prior to incubation with the nuclear extracts prepared from untreated or NGF-treated PC12 cells. The DNA-protein complexes were separated on a nondenaturing polyacrylamide gel. Bands containing complexed DNA (B) or free probe (F) were cut out, and the DNA was electroeluted. After piperidine cleavage, samples of DNA containing equal counts per minute were loaded and run on an 8% sequencing gel. DNA in lanes labelled P was partially methylated and cleaved with piperidine but was not incubated with extract. Arrowheads mark guanines less frequently methylated in DNA molecules forming DNA-protein complexes. The peripherin promoter sequence corresponding to these regions of the gels is shown at the left.

NGF for 1 week according to the method of Dignam et al. (14). We wished to obtain nuclear extract representing proteins present in fully differentiated PC12 cells. Therefore, treatment for 1 week with NGF was chosen to ensure that the majority of the PC12 cells harvested were fully differentiated. When the probe is incubated with nuclear extracts from undifferentiated PC12 cells, a slowly migrating DNA-protein complex predominates (large arrowhead in Fig. 3A), whereas a faster-migrating doublet is predominant in extracts from differentiated PC12 cells (arrows; compare lanes 1 and 4 in Fig. 3A). This doublet is present faintly in undifferentiated extracts (small arrowheads), perhaps reflecting the presence of a small fraction of active promoters accounting for the basal level of peripherin expression. However, there is no trace of the slowly migrating complex in extracts from differentiated PC12 cells. Therefore, there is a definite change in proteins complexed to this site as a result of NGF treatment. The specificity of these DNA-protein complexes is demonstrated by the ability of the unlabelled probe, but not nonspecific DNA fragments, to compete with the ^{32}P-labelled probe for protein binding (lanes 2, 3, 5, and 6).

Methylation interference footprinting of the NRE. To pinpoint more precisely the site of specific DNA-protein interaction, we performed methylation interference footprinting with the −245/−98 probe. Figure 3B demonstrates that probe molecules methylated at three G's on each strand were underrepresented in the retarded DNA-protein complex. These nucleotides define an 11-bp binding site, GGCAGGGCGCC (−178 to −168), which has imperfect dyad symmetry. Identical footprints are obtained with nuclear extracts from undifferentiated or differentiated PC12 cells, suggesting that the DNA binding protein may remain identical but that other components of the protein complex are altered between undifferentiated and differentiated states. Although the major protein-DNA contact points are clearly shown to be constant by methylation interference, DNase I protection or in vivo genomic footprinting potentially might reveal subtle changes in the boundaries of protein-DNA interactions after NGF treatment. The position of the footprinted sequence (−178 to −168) is precisely at the 5′ boundary (−179) of the shortest peripherin-CAT construct which still exhibits the negative phenotype.

To study the properties of proteins binding to the −178/−168 sequence, a synthetic oligonucleotide reproducing the footprinted sequence and surrounding nucleotides (−191 to −161) was synthesized, as well as oligonucleotides with mutations in the 5′ (RM1) or 3′ (RM2) contact residues or in both sides (RM3) of the footprinted site (Fig. 4A). When the ^{32}P-labelled WT oligonucleotide was incubated with extracts from undifferentiated and differentiated PC12 cells in the DNA mobility shift assay, it essentially reproduced the complexes formed with the −245/−98 probe (compare Fig. 4B and 3A). DNA mobility shift assays performed with nuclear extracts from PC12 cells treated for 3 h with NGF produced a slowly migrating complex identical to that seen with extracts from untreated PC12 cells (data not shown). This result is consistent with the delayed time course of

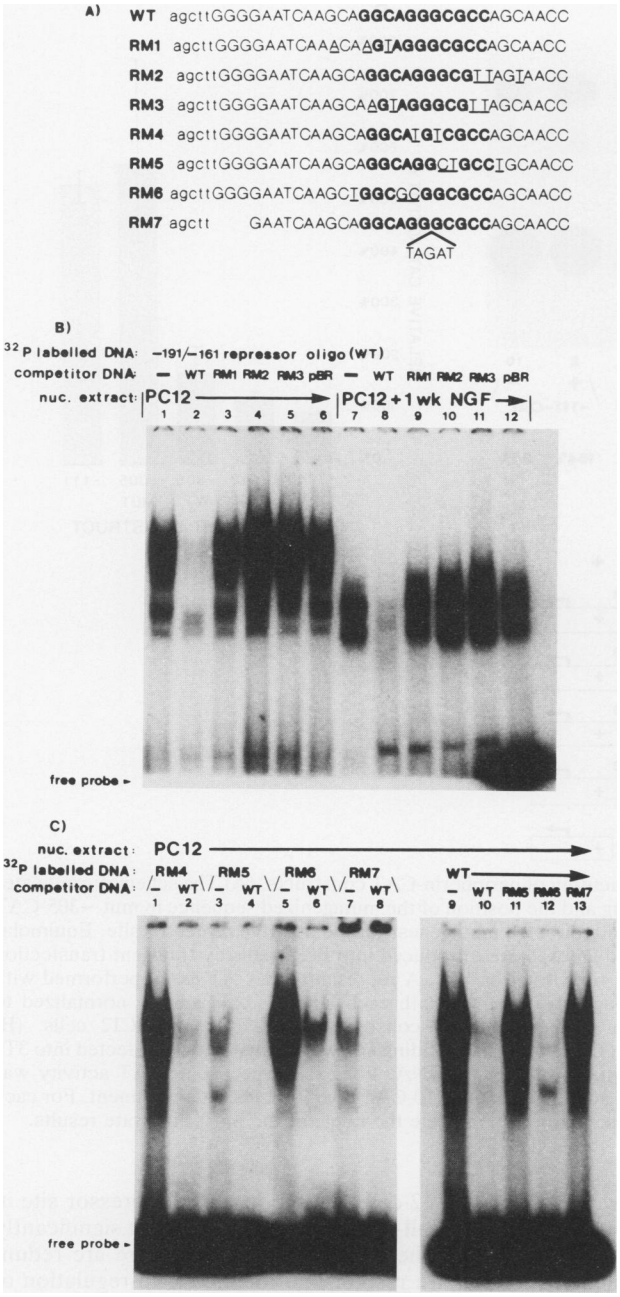

FIG. 4. Sequence requirements for binding of the putative repressor protein complex. (A) The sequences of the oligonucleotides used in DNA mobility shift assays. The peripherin promoter sequence included is from −191 to −161 relative to the transcriptional start site, except for RM7, which extends from −188 to −161. Bold letters indicate the putative repressor binding site defined by methylation interference footprinting. Underlined nucleotides have been mutated from the peripherin sequence (and are lightfaced if in the footprinted sequence). Lowercase letters are added *Hin*dIII linker sequences. (B) Nuclear extracts were prepared by the method of Dignam et al. (14) from untreated PC12 cells (lanes 1 to 6) or from PC12 cells grown in the presence of NGF for 1 week (lanes 7 to 12). DNA mobility shift assays were performed with the ^{32}P-labelled wild-type (WT) oligonucleotide in the presence of no competitor DNA (lanes 1 and 7), unlabelled WT oligonucleotide (lanes 2 and 8), the indicated unlabelled mutant oligonucleotides, RM1, RM2, and RM3 (lanes 3 to 5 and 9 to 11), or unlabelled pBR322-*Hae*III DNA fragments (lanes 6 and 12). The free probe has just migrated off the end of the gel in lanes 1 to 10. (C) DNA mobility shift assays were performed with nuclear extracts from untreated PC12 cells and the indicated ^{32}P-labelled mutant or WT oligonucleotide probe. Unlabelled WT oligonucleotide was included as a competitor in lanes 2, 4, 6, 8, and 10, and mutant unlabelled oligonucleotides were included as indicated in lanes 11 to 13. These gels were run in parallel, and autoradiography was performed for identical lengths of time.

induction of the peripherin gene. In competition experiments, unlabelled oligonucleotides bearing mutations in one or both sides of the footprinted site fail to compete with the wild-type labelled probe for protein binding (Fig. 4B, lanes 3 to 5 and 9 to 11). These results confirm that the DNA sequence defined by footprinting is specifically recognized by proteins present in PC12 cells which are altered during NGF-induced neuronal differentiation.

Control DNA mobility shift reactions performed with the cytoplasmic fraction of PC12 cell extracts prepared according to Dignam et al. (14) were identical with extracts from undifferentiated and NGF-differentiated PC12 cells and consisted of two weak retarded bands (data not shown). This finding excludes the possibility that the change in complexes seen in the nuclear extracts is due to real or artifactual loss of proteins from the nuclear into the cytoplasmic compartment. In addition, the possibility that the change to faster-migrating bands in the DNA mobility shift assay is due to general protein degradation is unlikely, since no major differences are seen in silver-stained sodium dodecyl sulfate-polyacrylamide gels of undifferentiated and differentiated nuclear extracts (data not shown).

Site-directed mutagenesis of the putative repressor site. Because the proteins specifically complexed to the footprint site are altered during NGF-induced differentiation, we next sought to confirm the functional importance of this putative repressor protein binding site by site-directed mutagenesis. The RM3 oligonucleotide, with mutations in the 5′ and 3′ contact nucleotides, was used as a primer for the mutagenesis of two peripherin-CAT plasmids: the fully NGF-inducible −3710-CAT construct and the poorly expressed −305-CAT construct (which lacks the distal positive element [DPE]). The ^{32}P-labelled RM3 oligonucleotide fails to form the slowly migrating complex with undifferentiated PC12 cell extract or a strong fast-migrating doublet with the differentiated PC12 cell extract (data not shown).

The results of introducing point mutations in the −178/−168 protein binding site are shown in Fig. 5A. Either the wild-type or the mutant peripherin-CAT plasmid was cotransfected with an RSV-β-galactosidase plasmid as an internal control for transfection efficiency, and CAT activity was normalized to β-galactosidase activity in protein extracts from transfected cells. Mutation of the putative repressor site in the context of the −305-CAT construct (mut −305-CAT) results in increased basal and NGF-induced expression similar to the expression of the constitutively active minimal promoter construct (−111-CAT; compare lanes 7 and 8 with 9 and 10 in Fig. 5A). Significantly, the basal activity of the mut −305-CAT construct is fivefold higher than the basal activity of the full-length −3710-CAT construct (compare lanes 2 and 8). Taken together, these data indicate that the −178/−168 protein binding site is essential for the repressive effect of the negative regulatory region defined by 5′ deletion analysis (−179 to −111). Therefore, we hypothesize that the protein binding to the footprint site functions as a repressor which negatively

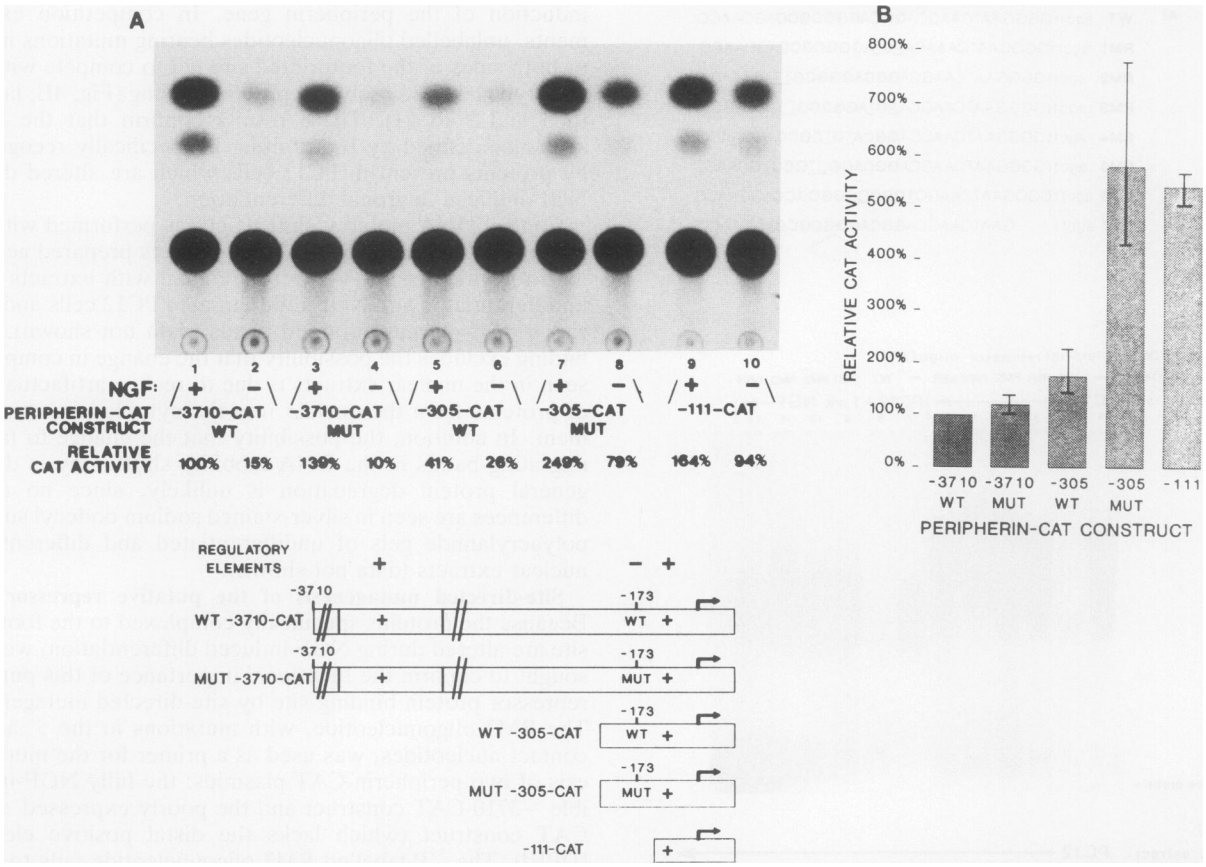

FIG. 5. Effects of mutation of the putative repressor binding site on the activity of peripherin-CAT constructs. (A) The schematic indicates the positive and negative regulatory elements defined by 5′ deletion mapping and the position of the mutagenized sequence in mut −305-CAT and mut −3710-CAT. The RM3 oligonucleotide sequence was used in the site-directed mutagenesis of the putative repressor site. Equimolar amounts of plasmid DNA, along with 5 μg of RSV–β-galactosidase plasmid DNA, were introduced into PC12 cells by transient transfection using electroporation. Parallel plates were untreated or treated with NGF for a total of 46 h. A representative CAT assay performed with protein extracts from the transfected cells is shown. The relative CAT activity listed beneath each lane is CAT activity normalized to β-galactosidase activity, expressed as a percentage of the activity of the −3710-CAT construct in NGF-treaetd PC12 cells. (B) Peripherin-CAT constructs with (MUT) or without (WT) mutations in the putative repressor binding site were transiently transfected into 3T3 cells by calcium phosphate precipitation along with 5 μg of RSV–β-galactosidase plasmid DNA. For each experiment, CAT activity was normalized to β-galactosidase activity and expressed as a percentage of the activity of the −3710-CAT construct in that experiment. For each construct, the mean of the results from two or more experiments is shown. Error bars indicate the range of the most disparate results.

regulates the proximal promoter element in undifferentiated PC12 cells.

However, mutagenesis of the repressor site in the context of the full-length promoter (mut −3710-CAT) has a less marked effect: the NGF-induced activity is similar to that of the wild-type −3710-CAT plasmid, and there is no increase in the low basal level of activity. This finding suggests that there are redundant sites in the peripherin promoter which function to maintain a low basal level of expression.

The NRE is active in nonneuronal cells. To ascertain whether the NRE is active in nonneuronal cells or whether it is a modulatory element active just in neuronal cells, we transfected the series of peripherin-CAT constructs with mutations at the repressor binding site into 3T3 cells. The activity of the −305-CAT construct mutated in the −178/−168 protein binding site is threefold higher than that of the wild-type construct and is similar to the activity of the −111-CAT construct (Fig. 5B). Therefore, the effect of the mutation at the repressor site on activity in 3T3 cells is similar to its effect in PC12 cells (compare Fig. 5B and 5A), implying that the NRE is active in nonneuronal cells. However, just as in PC12 cells, mutation of the repressor site in the context of the full-length promoter does not significantly increase activity. These data suggest that there are redundant sites which are responsible for the down-regulation of peripherin in nonneuronal cells.

Mutational analysis of the repressor binding site. Confirmation of the functional importance of the protein binding site in the NRE led us to gather additional information about the protein-DNA interactions at the repressor site. First, we performed DNA mobility shift assays in which oligonucleotides bearing mutations in various parts of the repressor binding site were used as ^{32}P-labelled probes (Fig. 4C, lanes 1 to 8) or as unlabelled competitors (lanes 11 to 13) in order to further define the sequence requirements for DNA-protein complex formation. Mutation of two central G's of the 11-bp repressor site (RM4) did not alter its binding characteristics (lanes 1 and 2); however, insertion of 5 bp in the center of the sequence drastically reduced formation of the slowly migrating complex unique to undifferentiated PC12 cell extract (lane 7) or the fast-migrating doublet in differentiated PC12 cell extracts (data not shown). With the addition of 5 bp in

the center of the sequence, the 5′ and 3′ contact nucleotides of the repressor site are separated by 1.5 helical turns instead of 1 helical turn. Therefore, this finding suggests that DNA-protein complex formation at the repressor site depends on interactions between proteins associating with each end of the palindrome which are normally situated on the same side of the helix.

Because we had noted a weak dyad symmetry to the footprinted repressor site, centered at position −173, we synthesized oligonucleotides which were perfect palindromes, matching either the 5′ (RM5) or 3′ (RM6) half of the sequence. The results demonstrate a functional asymmetry to this sequence, since the RM5 oligonucleotide forms only weak DNA-protein complexes when used as the probe in DNA mobility shift analysis (Fig. 4C, lanes 3 and 4) and is an ineffectual unlabelled competitor (lane 11), whereas the RM6 oligonucleotide forms a strong slowly migrating complex when incubated with undifferentiated PC12 cell extract (lanes 5 and 6) and is an effective competitor (lane 12). The multiple bands which appear with use of the putative repressor oligonucleotide in DNA mobility shift assays and the dependence of complex formation on the proper spacing between sites all point to the presence of multiple proteins at this site.

Comparison of protein complexes formed at the repressor site in different cell types. To determine the nature of complexes formed at the repressor site in nonneuronal cells, DNA mobility shift assays were performed by incubating the repressor site probe with nuclear extracts from a rat liver cell line (clone 9) and from undifferentiated or differentiated MEL cells (Fig. 6A). The nonneuronal cell extracts form a pattern of slowly migrating DNA-protein complexes similar, though not identical, to those formed with the undifferentiated PC12 extract. These results extend the correlation between the presence of a prominent slowly migrating complex and repression of peripherin expression. In addition, the change in the protein complex seen after NGF induction is not a nonspecific consequence of differentiation, since there is no appreciable difference in the shift pattern whether undifferentiated or differentiated MEL cell extract is used (Fig. 6A, lanes 6 to 8).

Mixing experiments show that a component in extracts from differentiated PC12 cells actively affects the complex formation at the repressor site. Experiments were performed to determine whether the differentiated or undifferentiated pattern of DNA-protein complexes is dominant when extracts from untreated PC12 cells or NGF-treated PC12 cells are mixed together prior to incubation with the repressor site probe. Figure 6B demonstrates that addition of increasing amounts of differentiated extract to a fixed amount of undifferentiated extract results in the loss of the most slowly migrating complexes unique to the undifferentiated extract (short arrows). The third retarded band (long arrow) increases in intensity to an extent greater than expected from the increasing component of differentiated extract. Therefore DNA-protein complexes formed with undifferentiated extract are dissociated in the presence of a component of the differentiated PC12 cell extract. Of note is the observation that the differentiated extract has a similar dominant effect when mixed with rat liver cell extract (Fig. 6B, lanes 8 and 9).

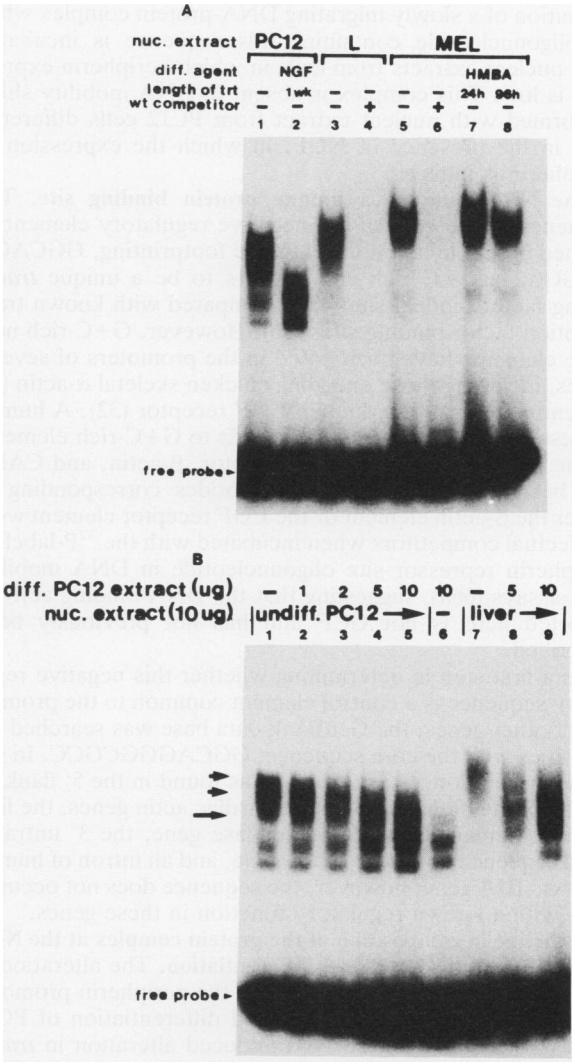

FIG. 6. Analysis of DNA-protein complex formation in extracts from different cell types and in mixing experiments. (A) DNA mobility shift assays were performed with the ^{32}P-labelled wild-type (WT) repressor site oligonucleotide and nuclear extracts from PC12 cells (untreated [lane 1] or treated with NGF for 1 week [lane 2]), clone 9 rat liver cells (L; lanes 3 and 4), or MEL cells (untreated [lanes 5 and 6] or treated with HMBA for 24 h [lane 7] or 96 h [lane 8]). Equal protein amounts of each extract were used. Unlabelled WT oligonucleotide competitor was present in reactions run in lanes 4 and 6. (B) The effect on complex formation of mixing extracts from undifferentiated and differentiated PC12 cells was assessed in DNA mobility shift assays performed by using the ^{32}P-labelled WT repressor site oligonucleotide. In lanes 1 to 5, 10 μg of nuclear extract from untreated PC12 cells was mixed with the indicated amounts of nuclear extract from PC12 cells treated for 1 week with NGF. The mixing was for 15 min on ice prior to incubation with the radiolabelled probe. Lane 6 shows complex formation with 10 μg of extract from NGF-treated PC12 cells alone. In lanes 7 to 9, 10 μg of nuclear extract from clone 9 rat liver cells was mixed with the indicated amounts of nuclear extract from NGF-treated PC12 cells.

DISCUSSION

Identification of an NRE in the peripherin promoter. We have identified a unique NRE in the peripherin promoter which contributes to the repression of peripherin expression in undifferentiated and nonneuronal cells. Mutation of the NRE in the context of the proximal 305 bp of the peripherin promoter causes an increase in activity of the reporter gene in both neuronal (PC12) and nonneuronal (3T3) cells. The function of this element as a repressor correlates with the

formation of a slowly migrating DNA-protein complex when an oligonucleotide containing this sequence is incubated with nuclear extracts from cells in which peripherin expression is low. This complex is absent in DNA mobility shifts performed with nuclear extract from PC12 cells differentiated in the presence of NGF, in which the expression of peripherin is induced.

The NRE contains a unique protein binding site. The sequence of the core of the negative regulatory element as defined by methylation interference footprinting, GGCAGG GCGCC, is G+C rich and appears to be a unique *trans*-acting factor binding site when compared with known transcription factor binding sites (46). However, G+C-rich negative elements have been noted in the promoters of several genes, including those encoding chicken skeletal α-actin (9), vimentin (17), and the human EGF receptor (32). A human repressor protein (GCF) which binds to G+C-rich elements in the promoters of the EGF receptor, β-actin, and CANP has been cloned (32). Oligonucleotides corresponding to either the β-actin element or the EGF receptor element were ineffectual competitors when incubated with the ^{32}P-labelled peripherin repressor site oligonucleotide in DNA mobility shift assays (66a), suggesting that the DNA-binding activity reported here is not GCF and has not previously been described.

As a first step in determining whether this negative regulatory sequence is a control element common to the promoters of other genes, the GenBank data base was searched for identities with the core sequence, GGCAGGGCGCC. In the sense orientation, the sequence was found in the 5′ flanking region of the human and mouse cardiac actin genes, the first exon of human tyrosine hydroxylase gene, the 3′ untranslated sequence of mouse NF-L gene, and an intron of human elastase IIIA gene; however, the sequence does not occur at sites with a known regulatory function in these genes.

A change in composition of the protein complex at the NRE occurs during NGF-induced differentiation. The alteration in the proteins binding to the NRE of the peripherin promoter which occurs during NGF-induced differentiation of PC12 cells is the first reported NGF-induced alteration in *trans*-acting factors controlling a late gene. Any model of the change in proteins which occurs at this site during NGF-induced differentiation must take into account two observations: (i) methylation interference footprinting demonstrates identical contact residues whether the probe is incubated with nuclear extract from undifferentiated or NGF-differentiated PC12 cells, and (ii) a slowly migrating complex is seen in DNA mobility shift assays using undifferentiated PC12 cell extract, whereas only a rapidly migrating complex is produced using nuclear extract from differentiated cells. Three types of changes in DNA-binding proteins could explain these findings. First, a ubiquitously expressed DNA-binding protein could bind to the site at all times, with effective repression achieved by the presence of an additional repressor protein(s) in the complex in undifferentiated cells. Second, a repressor protein contacting the site in the undifferentiated cell could be replaced by a smaller protein with an identical footprint when the gene is induced. Third, a common DNA-binding protein could bind at all times, but its modification by NGF-mediated events (e.g., phosphorylation) could change its perceived size in the DNA mobility shift assay and its function as a repressor.

Preliminary data support the first of these models. UV cross-linking studies to determine the molecular weight of the protein directly interacting with the DNA were performed by UV cross-linking of a bromodeoxyuridine- and ^{32}P-labelled repressor site oligonucleotide with crude nuclear extract from undifferentiated or differentiated PC12 cells. A 67-kDa protein with the appropriate specificity was labelled by using extract from either undifferentiated or differentiated PC12 cells (66a). (This molecular size is likely to be an overestimate of size due to the nucleotides which remain attached to the protein.) This finding is consistent with the model that a DNA-binding protein common to undifferentiated and differentiated PC12 cells binds directly to the repressor site.

Precedent for the interaction of a repressor protein with another protein which continually occupies the DNA binding site is found in the control of several well-studied yeast genes. The upstream activating region of the yeast *GAL* genes is bound by the GAL4 transcription factor at all times, but in conditions lacking galactose, *gal80* contacts *gal4* and inhibits its ability to activate transcription (31). In the control of the yeast mating-type locus, the cell-type-specific α2 repressor protein must form a complex with the ubiquitous GRM/PRTF protein at the **a**-specific operator in order to repress **a**-specific gene expression in α cells (34, 35).

Identification of the factors binding to the repressor site will be necessary for a full understanding of the mechanism by which NGF treatment of PC12 cells alters the repressor protein complex. However, some insight into mechanism is gained from the results of the DNA mobility shift assay in which extracts from undifferentiated and differentiated PC12 cells were mixed prior to incubation with the repressor site probe (Fig. 6B). The dominance of the differentiated pattern of retarded bands indicates that a component of the differentiated extract prevents the formation of DNA-protein complexes which would otherwise form with protein in the undifferentiated extract. This result has multiple interpretations, including the possibility that an undifferentiated cell factor (repressor) is modified by an activity present in differentiated PC12 cells. If the alteration in complexes at the NRE during NGF-mediated differentiation was merely due to cessation of synthesis of the repressor protein, then mixing of the extracts would have produced a summation of the complexes seen in each extract.

Role of the NRE in tissue-specific expression of peripherin. In addition to modulating the response of the peripherin gene to NGF in a neuronal cell type, the NRE also appears to function to repress peripherin gene expression in nonneuronal cells (3T3 cells). Mutation or deletion of the NRE has an effect on the activity of peripherin-CAT constructs in 3T3 cells similar to that in untreated PC12 cells. This correlates well with the presence of slowly migrating DNA protein complexes in DNA mobility shift assays which incubate the repressor site probe with extracts of either nonneuronal cells or undifferentiated PC12 cells. Therefore, negative regulation by the NRE may contribute to the tissue-specific expression of the peripherin gene. In nonneuronal cells, failure to overcome the repression mediated by the NRE may be due to inability to dissociate the repressor protein complex from the NRE and/or inability to activate the distal positive element (DPE). It is noteworthy that NREs in the promoters of SCG10 (55) and the type II sodium channel gene (49) contribute to the neuronal-specific expression of these NGF-responsive genes as well.

However, other mechanisms must also contribute to the tissue-specific expression of peripherin. Although −3710-CAT activity in 3T3 cells is 25-fold less than the activity in NGF-differentiated PC12 cells, it is only 4-fold less than the activity in undifferentiated PC12 cells. A basal level of expression of peripherin in undifferentiated PC12 cells is

expected from Northern (RNA) blot analysis of endogenous peripherin gene expression (42); however, no expression is detected in fibroblasts (22, 41) by in situ hybridization. Therefore, the small but significant basal level of expression of the transfected peripherin-CAT construct in 3T3 cells indicates that complete shutoff of the gene in nonneuronal cells may depend on negative elements lying outside of the 3,710 bp of 5′ flanking sequence included in the largest peripherin-CAT construct. Sarkar and Cowan (62) found an NRE in the first intron of the gene encoding another intermediate filament protein, GFAP, which is important for its tissue-specific expression in glial cells. Alternatively, the basal expression in 3T3 cells may be an artifact of the transient transfection system; Wuenschell et al. (72) showed that a construct containing 3.7 kb of the neuronal-specific SCG10 gene promoter was expressed to a small degree in HeLa cells in transient transfections, although the same construct showed absolute tissue specificity of expression in transgenic mice. Certainly, if tissue-specific regulation of the endogenous gene is at the level of chromatin conformation, this will not be recapitulated in transient transfection assays.

Evidence for additional NREs in the peripherin promoter. We have established the importance of the protein binding site within the NRE in two ways. First, we have shown that the protein complex at this site changes during NGF-induced neuronal differentiation, and second, we have clearly shown that the protein binding site has a functional role in repressing peripherin expression by mutagenesis of the site in the context of the −305-CAT construct. The observation that mutation of the NRE in the context of 3.7 kb of 5′ flanking sequence (−3710-CAT) has little effect on promoter activity in either PC12 or 3T3 cells suggests that there are redundant NREs in the peripherin promoter which function to maintain a low basal level of expression of the gene in undifferentiated and nonneuronal cells. Multiple NREs have also been mapped in the promoters of the chicken lysozyme gene (5), the insulin II gene (11), and the skeletal α-actin gene (9). Teleologically, this multiplicity may offer additional modulation points at which the peripherin gene expression can be fine-tuned in response to various environmental stimuli or in different cell types.

A model for induction of peripherin gene expression by NGF. The following two-step model for how NGF induces peripherin gene expression accounts for our experimental observations (Fig. 7). The proximal promoter, as defined by the −111-CAT truncation, is constitutively active in isolation but is subject to regulation by NGF via interactions with upstream elements. At least two upstream regulatory regions exist: the NRE, centered at −173, and the DPE, between −2660 and −2290. Following NGF treatment (Fig. 7A), a repressor protein dissociates from the complex at the NRE, a change which is visualized in DNA mobility shift assays with the isolated repressor site oligonucleotide as loss of the slowly migrating complex. Following dissociation of the repressor, the DPE overcomes the residual negative effect of the complex remaining at the NRE (the fast-migrating doublet visualized in DNA mobility shift assays with differentiated PC12 cell extract). This second step of activation may be the result of an NGF-induced positive *trans*-acting factor binding to the DPE. The model suggests that full relief of repression in the presence of NGF depends on the influence of the DPE as well as release of a negative factor from the NRE. This two-step model of activation explains the inability of the wild-type −305-CAT construct to be fully activated by NGF (Fig. 7B). When only the NRE is present, even though the release of a negative factor from the NRE presumably still occurs, the promoter remains inactive since the DPE-NRE interaction cannot occur. However, removal of the NRE altogether by mutation (mut −305-CAT; Fig. 7C) or by truncation (−111-CAT; Fig. 7D) releases the proximal promoter from all negative control and results in constitutive activity.

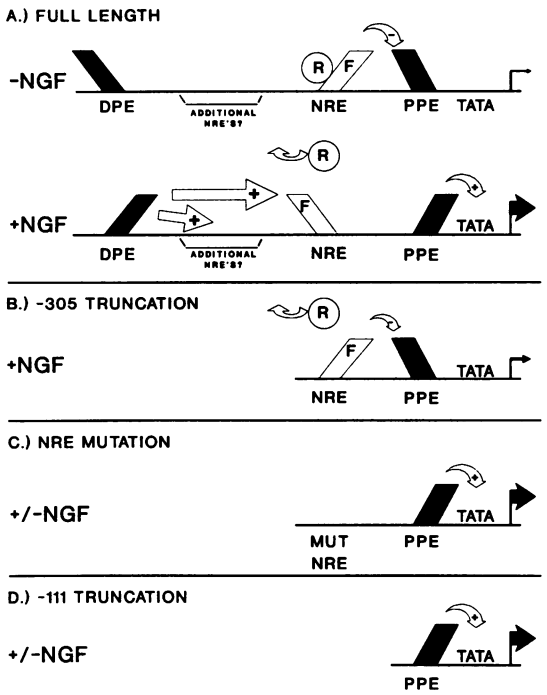

FIG. 7. Model for the induction of peripherin gene expression by NGF. (A) Two hypothetical states of the peripherin promoter are shown: the basal (−NGF) and the NGF-induced (+NGF) states. We hypothesize that in the undifferentiated PC12 cell, the protein complex (R+F) situated at the NRE has a negative effect on a constitutive factor binding to the proximal positive element (PPE). We hypothesize that the gene is induced by a two-step mechanism in the NGF-differentiated cell. First, NGF causes a repressor molecule, R, to be released from the NRE complex, while factor F remains at this site. This is reflected in the change from slowly migrating (R+F) to fast-migrating (F) complexes in the DNA mobility shift assay. The factor binding to the DPE, which may be modified or induced by NGF, is able to overcome the residual negative effect of the remaining complex (F) at the NRE. (B) The low activity of the −305-CAT construct in NGF-treated PC12 cells suggests that the DPE is necessary for full relief of repression. In C and D, the mutant −305-CAT construct and the −111-CAT construct are active in either untreated or NGF-treated PC12 cells as a result of the unopposed positive activity of the proximal positive element in the absence of factors binding to the NRE.

Precedent for an inducible gene being activated by relief of repression is found in the response of the ovalbumin gene to steroid hormones. The ovalbumin promoter has an arrangement of regulatory elements similar to that of the peripherin promoter, with an NRE situated 5′ of a constitutively active proximal promoter element (60). The upstream steroid-dependent regulatory element induces the ovalbumin gene in the presence of hormone by derepressing the NRE (19, 63). Consistent with this model of derepression is the fact that the response to steroid is transferred to a heterologous promoter only when the steroid-dependent regulatory element and NRE are both transferred as a unit (19, 63). The effects of the

analogous elements of the peripherin promoter on a heterologous promoter are currently being investigated.

In summary, we have shown that NGF activates peripherin gene expression in part by relieving the repression mediated by an NRE. This activation is associated with alteration of proteins binding to a unique DNA sequence in the NRE. Our effort is now directed toward identification of the presumptive repressor protein(s) in order to understand how it is regulated by NGF. In addition, we shall determine whether NGF induces a positive *trans*-acting factor which mediates the derepression from the DPE. Understanding how NGF provides these activating signals will add to our knowledge of the pathway by which NGF induces genes necessary for the neuronal phenotype.

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